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## Comparative analysis of the fusion efficiency elicited by the envelope glycoprotein V1–V5 regions derived from human immunodeficiency virus type 1 transmitted perinatally

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### Abstract

Understanding the properties of viruses preferentially establishing infection during perinatal transmission of human immunodeficiency virus type 1 (HIV-1) is critical for the

development of effective measures to prevent transmission. A previous study demonstrated that the newly transmitted viruses (in infants) of chronically infected mother–infant pairs (MIPs) were fitter in terms of growth, which was imparted by their envelope (Env) glycoprotein V1–V5 regions, than those in the corresponding chronically infected mothers. In order to investigate whether the higher fitness of transmitted viruses was conferred by their higher entry efficiency directed by the V1–V5 regions during perinatal transmission, the fusogenicity of Env containing V1–V5 regions derived from transmitted and non-transmitted viruses of five chronically infected MIPs and two acutely infected MIPs was analysed using two different cell–cell fusion assays. The results showed that, in one chronically infected MIP, a higher fusion efficiency was induced by the infant Env V1–V5 compared with that of the corresponding mother. Moreover, the V4–V5 regions played an important role in discriminating the transmitted and non-transmitted viruses in this pair. However, neither a consistent pattern nor significant differences in fusogenicity mediated by the V1–V5 regions between maternal and infant variants was observed in the other MIPs. This study suggests that there is no consistent and significant correlation between viral fitness selection and entry efficiency directed by the V1–V5 regions during perinatal transmission. Other factors such as the route and timing of transmission may also be involved.

## Introduction

Mother-to-child transmission of human immunodeficiency virus type 1 (HIV-1) remains the major route of infection for children in endemic regions ([Alcântara et al., 2012](#); [Steain et al., 2006](#)). This is especially significant in sub-Saharan Africa where >90% of infected children under the age of 15 live and where the majority (95%) of new paediatric infections

occurred, and where therapeutic intervention is still not widely available ([Ahmad, 2005](#); [Luzuriaga, 2007](#); [Zhang et al., 2010b](#)). Moreover, HIV-1 subtype C is the most prevalent subtype and accounts for >60% of infections in this epidemic region. Given the absence of a prophylactic vaccine or universal access to preventative treatment in these developing countries, a clear understanding of the characteristics of preferentially transmitted viruses is critical for the development of effective measures to reduce rates of mother-to-child transmission.

Studies in multiple cohorts, across several HIV-1 subtypes, have shown that only a limited number of viruses within the viral quasispecies are transmitted during sexual transmission ([Derdeyn et al., 2004](#); [Keele et al., 2008](#); [Zhu et al., 1993](#)) or perinatal transmission ([Dickover et al., 2001](#); [Kishko et al., 2011](#); [Scarlati et al., 1993](#); [Verhofstede et al., 2003](#); [Wolinsky et al., 1992](#)). In fact, there are a number of host and viral factors that have been suggested to affect the perinatal transmission of specific viral species, including host neutralizing antibodies ([Barin et al., 2006](#); [Dickover et al., 2006](#); [Wei et al., 2003](#)), the timing of transmission ([Dickover et al., 2001](#)) and the R5 tropism of HIV-1 ([Kishko et al., 2011](#); [Verhofstede et al., 2003](#); [Wolinsky et al., 1992](#)). However, there is no consensus on the mechanism that accounts for the observed selective transmission bottleneck, implying a complexity during perinatal transmission and underscoring the need to explore further the genetic and biological determinants of this transmission bottleneck.

We have centred our efforts on identifying cases of mother-to-child transmission in a cohort in Zambia as part of ongoing efforts to understand transmission and disease progression in HIV-1 subtype C infection. We found previously that there was a restricted diversity in the infant viral

quasispecies during perinatal transmission from mothers who were chronically infected ([Zhang et al., 2010b](#)). Moreover, in the five chronically infected mother–infant pairs (MIPs) analysed here, the infant viruses were selected to have higher ex vivo fitness, as imparted by the V1–V5 regions of the surface gp120 glycoprotein, than viruses of their corresponding mothers ([Kong et al., 2008](#)). In contrast, we did not observe the same genotype and phenotype selective transmission in our acutely infected pairs ([Hoffmann et al., 2008](#); [Kong et al., 2008](#)).

Glycoprotein gp120 directs HIV-1 entry into host cells by binding initially to the CD4 receptors of target cells, resulting in dramatic conformational changes in gp120 that expose the binding site for a secondary co-receptor. Upon binding of gp120 to the co-receptor, further conformational changes in gp120 lead to a gp41-mediated membrane fusion process that delivers the viral core into the cytoplasm ([Checkley et al., 2011](#)). Given that gp120 has an important impact on viral entry, we therefore explored further the possibility that the V1–V5 regions of gp120 could affect gp41-mediated fusion to confer higher replicative fitness to the infant viruses. Two different fusion assays were employed to compare the fusogenicity elicited by the V1–V5 regions derived from infant and maternal variants of the seven Zambian MIPs in this study to determine whether the Env V1–V5 regions obtained from the infants and their mothers affected fusion differentially to contribute to their differences in viral fitness and subsequently their transmission.

## Results

### Characteristics of the seven MIPs

As summarized in [Table 1](#), the maternal age at delivery was between 19 and

31 years. The mode of delivery of all MIPs was vaginal except for MIP 2617, which was by caesarian section. The mothers of chronically infected MIPs were known to be HIV-1 positive at the time of delivery and were likely to have acquired HIV-1 infection heterosexually, whilst their infants were HIV-1 negative at birth, suggesting that the infants were infected either intrapartum or post-partum. For the acutely infected MIPs, mothers and infants were found to have seroconverted at the same time point after birth, suggesting that the infants were infected through breast-feeding. All the mothers were asymptomatic without any clinical signs of immunosuppression, and the infants were all breast-fed and drug naïve. Infants of pairs 2617, 2669, 2873 and 1449 were considered rapid progressors, as they died within the first year of life due to apparent HIV-related complications.

**Table 1.**  
Summary of clinical information for the MIPs included in this study  
na, Not applicable.

Subject	Age (years) <sup>a</sup>	Birth weight (kg)	Mode of delivery	Breast-feeding analysed (months) <sup>b</sup>	Time point analysed (months) <sup>c</sup>	No. of clones analysed	Clinical information
MIP 1084							
II Mother	28	na	na	na	MD	3	Found to be HIV-1 positive at the time of delivery, clinically asymptomatic.
II Infant, male	na	3400	Vaginal	Yes	6	3	After following for more than 6 years, remained clinically asymptomatic.

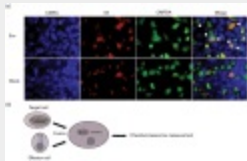
[See full table](#)

**Table 1. . Summary of clinical information for the MIPs included in this study** <sup>na</sup>, Not applicable.

Fusogenic properties of Env containing V1–V5 regions from maternal and infant HIV-1 variants of chronically and acutely infected MIPs

To determine whether the differences in replicative fitness conferred by the V1–V5 regions between transmitted and non-transmitted variants of our chronically infected MIPs were related to different viral entry efficiencies, we compared the entry efficiency mediated by the V1–V5 regions derived from the infant and maternal variants. A common surrogate assay of HIV-1 fusion and entry is the cell–cell fusion assay, where effector cells expressing

HIV-1 Env (gp120/gp41) are co-cultured with target cells expressing the CD4 receptors and CCR5/CXCR4 co-receptors ([Deng et al., 1996](#)). To measure cell–cell fusion activity, we implemented two different assays. The first was a fluorescent probe transfer system using COS-1 cells as effector cells, which were transfected with the Env expression plasmids and loaded with 7-amino-4-chloromethylcoumarin (CMAC; blue), and TZM-bl cells loaded with the lipophilic octadecyl (C18) indocarbocyanine probe DiI (red) and 5-chloromethylfluorescein diacetate (CMFDA; green) as the target. The fused cells containing all three dyes were then quantified using fluorescence microscopy ([Fig. 1a](#)). In parallel, a second assay was carried out using COS-1 cells transfected with Env expression plasmids and TZM-bl cells containing the luciferase reporter gene under the control of the HIV-1 long-terminal-repeat promoter (indicator cells) ([Fig. 1b](#)). A luciferase signal was emitted by the fused cells and used to measure cell–cell fusion mediated by Env.



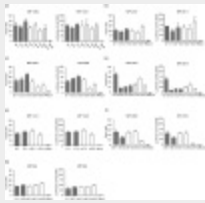
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**Fig. 1.** . Fusion assays. (a) Fluorescent microscopic images of cell–cell fusion mediated by Env using a dye-transfer-based assay. Effector (COS-1) cells expressing Env were labelled with CMAC (blue). Target (TZM-bl) cells were co-labelled with DiI (red) and CMFDA (green). Fluorescence images for each dye were acquired as shown. Fused cells are depicted by arrowheads. (b) Schematic representation of the reporter gene-based cell–cell fusion assay. TZM-bl cells were used as target cells and luciferase activity was measured to indicate fusion.

We first analysed the chimaeric Env containing the viral V1–V5 regions of the patients for their ability to induce cell–cell fusion using the fluorescent probe transfer assay. The comparison of fusion efficiency between maternal and infant variants of each MIP is shown in [Fig. 2](#), left graphs). The results



suggested that, in general, most of the Env proteins containing the V1–V5 regions of the transmitted variants exhibited comparable fusion efficiency to the non-transmitted maternal variants derived from the five chronically infected MIPs and two acutely infected MIPs. We also used the reporter gene-based fusion assay to substantiate these results further ([Fig. 2](#), right panels). Similar results were observed when compared with the dye-transfer assay. Moreover, none of the mean fusion rates of the chimaeric Env containing infant V1–V5 regions was statistically different from those of the mothers of the MIPs (Table S1, available in JGV Online).

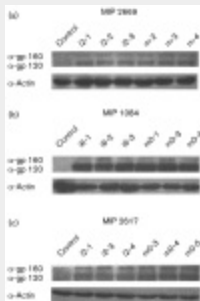


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**Fig. 2.** . Comparison of the fusion efficiency between the infant and maternal variants from MIP 1084 (a), MIP 2617 (b), MIP 2669 (c), MIP 2873 (d), MIP 1449 (e), MIP 2660 (f) and MIP 834 (g) using a dye-transfer-based assay (left graphs) and a reporter gene-based assay (right graphs). COS-1 cells were transfected with Env-expressing plasmids and used as effector cells. TZM-bl cells were used as target cells. Maternal samples were collected at the delivery, indicated as m0. Infants samples were collected at the time of the first HIV PCR-positive result after birth, which for most infants was at 2 months after birth (i2), for some was at 4 months (i4) and for some was at 6 months (i6). The number after the dash indicates the clone number. Results are shown as means $\pm$ sd from three independent experiments.

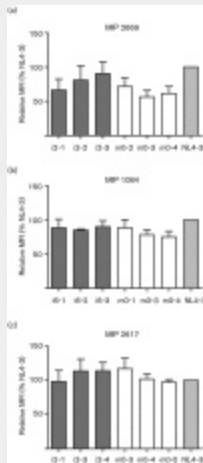
However, we noted that all infant viral Env V1–V5 regions of MIP 2669 led to higher fusion efficiency than most of the maternal V1–V5 regions except for MIP 2669 m0-2, which had a fusion efficiency comparable to that of the infant ([Fig. 2c](#)). To exclude the possibility that the expression level of the chimaeric Env protein containing patients' V1–V5 regions contributed to differences between maternal and infant variants, we first tested the cell-associated expression of Env glycoproteins containing the V1–V5 regions of MIP 2669 and two other representative chronically infected MIPs (MIP

1084 and MIP 2617). As shown in [Fig. 3](#), we observed similar profiles for the cell-associated Env glycoproteins. As synthesis of Env glycoproteins appeared to be normal, we next examined the surface expression of Env containing the V1–V5 regions derived from patients to determine whether they were transported to and retained normally in the plasma membrane. In this analysis, the cell-surface expression levels of all the Env glycoproteins containing the infant and maternal V1–V5 regions of MIP 2669, MIP 1084 and MIP 2617 did not differ significantly ([Fig. 4](#)). These results indicated that the V1–V5 regions of gp120 did not lead to viral envelope expression differences to discriminate the transmitted and non-transmitted viruses. Thus, in general, there seemed to be no consistent and significant differences in fusogenicity induced by the V1–V5 regions between the infant and maternal variants, although for MIP 2669 it appeared that the infant V1–V5 regions induced a higher fusion efficiency than most of the maternal Env glycoproteins.



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**Fig. 3. .** Expression profiles of Env glycoproteins containing the V1–V5 regions derived from MIP 2669 (a), MIP 1084 (b) and MIP 2617 (c). COS-1 cells transiently transfected with simian virus 40 (SV40)-based Env expression vectors containing the V1–V5 regions derived from patients were collected at 48 h post-transfection, lysed and analysed by SDS-PAGE. Detection of actin was used as a loading control.



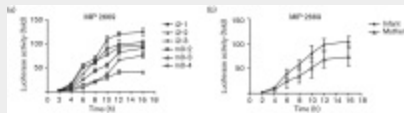
[View larger version](#)

**Fig. 4.** . Cell-surface levels of Env glycoprotein containing the V1–V5 regions derived from three chronically infected MIPs. COS-1 cells transfected with Env expression plasmids containing the V1–V5 regions derived from patients were fixed at 48 h post-transfection, labelled with anti-gp120 HIV antibody and analysed by flow cytometry. The surface levels of Env derived from MIP 2669 (a), MIP 1084 (b) and MIP 2617 (c) are shown as the relative mean fluorescence index (MFI) compared with that of strain NL4-3 Env. Results are shown as means±SD from three independent experiments.

Env containing maternal V1–V5 regions shows delayed cell–cell fusion kinetics when compared with that of their infants from MIP 2669

As MIP 2669 showed a trend where the infant V1–V5 regions induced a higher fusion efficiency than most of the maternal V1–V5 regions, we measured the kinetics of cell–cell fusion induced by the V1–V5 regions using a reporter gene-based assay. As shown in [Fig. 5\(a\)](#), Env containing maternal V1–V5 regions (m0-3 and m0-4), which showed a low fusion efficiency ([Fig. 2c](#)) exhibited a lag time of about 2 h prior to the onset of fusion compared with the infant Env glycoproteins, after which they displayed a steady increase in luciferase activity, reaching a plateau at about 12 h, whilst m0-2 displayed a similar fusion profile to the infant Env glycoproteins. We then compared the mean values of the fusion kinetics of all Env containing infant and maternal V1–V5 regions ([Fig. 5b](#)). Env containing infant V1–V5 regions showed a higher overall initial rate between 4 and 6 h (14-fold versus eightfold  $\text{h}^{-1}$ , respectively) than maternal variants in inducing cell–cell fusion. Moreover, the infant and maternal fusion kinetics were distinguishable from each other by reaching different peak fusion levels. In addition, the time to half-maximal fusion of Env

containing infant V1–V5 regions was shorter than that of the mothers (7.4 versus 8.3 h, respectively). In conclusion, for MIP 2669, the Env glycoprotein containing the V1–V5 regions of infant variants exhibited a trend of faster fusion kinetics when compared with Env containing the V1–V5 regions of the maternal variants.



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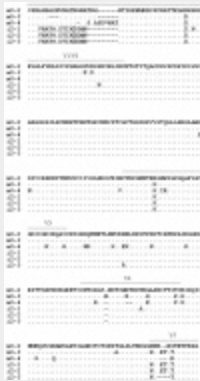
**Fig. 5. .** Kinetics of cell–cell fusion mediated by the V1–V5 regions of infant and mother derived from MIP

2669. COS-1 cells transfected with Env containing the V1–V5 regions from chronically infected MIP 2669 were mixed with TZM-bl indicator cells and co-cultured for 2, 4, 6, 8, 10, 12 and 16 h before measuring the luciferase activity. (a) The relative luciferase activities of each infant and maternal variant were calculated at each time point and plotted as a single line. Results are shown as means $\pm$ SD of at least three independent experiments. (b) Relative luciferase activities of all maternal and infant variants. Results are shown as means $\pm$ SEM of infant and mother.

The higher fusogenicity induced by the V1–V5 regions of infant variants of MIP 2669 compared with the maternal variants is determined mainly by the V4–V5 regions

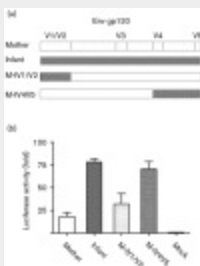
To investigate why Env containing maternal V1–V5 regions of MIP 2669 exhibited a low fusion efficiency compared with that of the infant variants, we first compared the sequence of the V1–V5 regions of all the infant and maternal variants of MIP 2669. Inspection of the variation between the infant and maternal variants revealed that the most variable areas were the V1–V2 loop, the V3 loop, the 5' end of C3 and the variable loops V4 and V5 ([Fig. 6](#)). To determine which of these regions might contribute to the higher fusion efficiency of the infant Env, the V1–V2 and V4–V5 regions of the maternal variants were replaced with those of the infant variants ([Fig. 7a](#)). As shown in [Fig. 7\(b\)](#), the low fusion efficiency of Env containing maternal V1–V5 regions was moderately rescued when the V1–V2 regions were

replaced by the infant V1–V2 regions. However, a fusion efficiency equivalent to that of the infant variants was observed when the maternal V4–V5 region was replaced by the V4–V5 region of the infant variants. In conclusion, the V4–V5 regions may play an important role in affecting fusion efficiency and distinguishing the differences between the maternal and infant variants of MIP 2669.



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**Fig. 6. .** Alignment of deduced amino acid sequences of the Env glycoprotein spanning the V1–V5 regions derived from chronically infected MIP 2669. Amino acid identity (.), insertions/deletions (–) and substitutions are indicated.



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**Fig. 7. .** Identification of V4–V5 regions responsible for the high fusion efficiency of infant variants compared with the maternal variants in MIP 2669. (a) Schematic representation of chimaeric Env constructs of MIP 2669. (b) Comparison of the fusion efficiency of wild-type mother and infant and chimaeric Env using a reporter gene-based assay.

## Discussion

Our overall results suggested that, in general, most of the chimaeric Env containing the V1–V5 regions derived from the infant variants exhibited a fusion efficiency comparable to that of the maternal variants in chronically or acutely infected MIPs. It seems likely that the selective higher fitness in chronically infected MIPs during perinatal transmission is not driven

exclusively by selection of viruses with a significantly higher entry efficiency mediated by the V1–V5 regions from the maternal viral population. These results contrast with those reported by [Marozsan et al. \(2005\)](#) studying the replication fitness of different HIV-1 subtypes, who demonstrated that a subtype B isolate had a higher replicative fitness than a subtype C isolate, and that its higher fitness correlated with a higher fusion ability imparted by the V1–V5 region. These discrepancies may be due to inter-subtype differences in the Env V1–V5 regions of subtype C viruses. Moreover, in our study, the transmitted and non-transmitted viruses were from the same viral quasispecies ([Zhang et al., 2010b](#)). Alternatively, although the fusion ability induced by the Env V1–V5 regions between transmitted and non-transmitted viruses was comparable, Env harbouring V1–V5 regions of transmitted viruses may act more subtly to overcome the non-transmitted viruses during perinatal transmission. Indeed, this has been observed in our own and other previous studies, where the differences in viral fitness could only be differentiated by direct competition ([Martinez-Picado et al., 2006](#); [Zhang et al., 2010a](#)).

However, we also noted that, in one of our chronically infected pairs, MIP 2669, where the infant was a rapid progressor, most of the Env glycoproteins containing maternal V1–V5 regions exhibited a lower fusion efficiency than Env containing the infant V1–V5 regions and displayed delayed cell–cell fusion kinetics compared with the infant variants. Sequence comparison of the maternal and infant variants of MIP 2669, and results obtained from chimaeric Env variable region clones, demonstrated that the V4–V5 regions of the Env glycoprotein may undergo selective changes in a non-random fashion to produce the transmitted infant variants harbouring higher fusion efficiencies. Our results with MIP 2669 support our previous study demonstrating that high levels of non-synonymous

variation occurred within the V1/V2 loops, V4 and V5 regions, and that potential N-glycosylation site changes also generally occurred within the V1, V2 and V4 regions ([Zhang et al., 2006, 2010b](#)). In addition, a previous study also suggested that the V4 region was involved in Env conformation change and glycan packing ([Wei et al., 2003](#)).

A number of gp120 structures have provided a wealth of information on the mechanism of gp120 interactions with CD4, co-receptor and gp41 ([Chen et al., 2005](#); [Pancera et al., 2010](#); [Wyatt et al., 1998](#)). These studies also suggested that, in addition to binding to receptors, gp120 plays an important role in the viral membrane to subsequently affect viral fusion with target cells. Several studies have demonstrated that mutations impacting on the stability of CD4-binding sites and several residues in the V3 domain could contribute to final viral fusogenicity ([Gray et al., 2011](#); [Rossi et al., 2008](#); [Sterjovski et al., 2007, 2011](#)). We found that the V4–V5 regions affected the fusion efficiencies of Env derived from the MIP 2669, even though V4–V5 has not been shown previously to participate in CD4 or co-receptor binding. It is possible that the V4–V5 region of the infant Env variants may confer subtle conformational changes in gp120 to induce more-efficient fusion. Further studies will be needed to elucidate fully the specific determinants within the V4–V5 region that enable it to induce a higher fusogenicity. Although MIP 2669 showed a trend of selection of special V4–V5 regions to lead to a final higher fusogenicity and faster fusion kinetics, we could not exclude the possibility that this was sample dependent. Therefore, more samples with this sequence specificity will be needed to confirm our observations further. Nevertheless, our findings still provide some insight into the features of gp120 that could be associated with its enhanced fusion ability and how this may contribute to the bottleneck selection during perinatal transmission.

It has been reported that there is selection of single or minor variants to be transmitted perinatally ([Briant et al., 1995](#); [Dickover et al., 2001](#)). For MIP 2669, the transmission of a single maternal variant appeared to have occurred partially due to the selection of variants that harboured the V1–V5 regions to induce higher Env fusion efficiency, whilst for the other chronically infected MIPs, there could be other factors involved including the timing and route of transmission ([Briant et al., 1995](#); [Dickover et al., 2001](#); [Kwiek et al., 2008](#)) that may contribute to the seeding of minor variants from the maternal quasispecies to initiate infection. Unfortunately, the precise timing of transmission in our MIPs could not be determined, and transmission could have occurred either late in utero or via early breast-feeding, instead of being true intrapartum infection.

There are several lines of evidence to suggest that HIV-1 transmembrane subunit gp41 modulates efficient intracellular transport and processing of Env precursor gp160 and cell-surface expression either by glycan components or by the cytoplasmic tail region ([Bültmann et al., 2001](#); [Dash et al., 1994](#); [Fenouillet & Jones, 1995](#)). Moreover, mutations of gp120 could also lead to impaired cleavage and export from the endoplasmic reticulum of gp160 ([Guo et al., 1990](#); [Willey et al., 1991](#)). Regarding our chimaeric Env containing the variable V1–V5 regions derived from patients, which shared the same signal peptide that targets Env to the endoplasmic reticulum, as well as the same gp41 sequence, we wanted to investigate whether the expression level of the chimaeric Env containing the V1–V5 regions of the patients contributed to differences between transmitted and non-transmitted viruses by testing cell-associated and cell-surface expression levels. No significant differences in the two modes were observed either in MIP 2669 or in two other chronically infected MIPs. These results, together with our previous pulse–chase analysis of the



kinetics of synthesis and processing of these Env expression constructs ([Zhang et al., 2010a](#)), suggest that the variability of gp120 V1–V5 regions does not contribute to Env cellular biosynthesis, processing or stability after gp120 reaches the plasma membrane to distinguish between maternal (non-transmitted) and infant (transmitted) viruses.

One of the limitations in this study was that single-genome amplification was not performed on these samples, as they were amplified previously from the pooling of four to five diluted bulk PCRs, rather than from the bulk PCR one time only. There is the possibility that PCR recombination in vitro may have occurred to produce env genes that were not representative in vivo. However, our previous phylogenetic analysis showed that the number of unique sequences was large and was similar at each time point, suggesting none of the clones was the result of in vitro PCR recombination ([Kong et al., 2008](#)).

In conclusion, for the MIPs analysed, the fusogenicity elicited by the V1–V5 regions of Env may not be the exclusive factor that can impact on fitness selection during perinatal transmission, although a trend towards higher fusogenicity induced by the V1–V5 regions of transmitted infant variants was observed in one chronically infected MIP analysed. Moreover, neither Env biosynthesis nor cell-surface expression was predictive of virus transmissibility in our cohort. Other virus properties, such as transmission routes and timing, may also be involved.

## Methods

Patient information and sample collection.

Seven MIPs (pairs 1084, 2617, 2669, 2873, 1449, 2660 and 834) were

recruited into the study. This study was approved by the University of Nebraska-Lincoln Institutional Review Board and the University of Zambia, School of Medicine Research Ethics Committee. All study participants or their parents provided written informed consent for the collection of samples and subsequent analysis. Venous blood was collected from the mother before delivery and from the infant within 24 h of delivery and at subsequent time points. The mothers of five MIPs (pairs 1084, 2617, 2669, 1449 and 2873), which were defined as chronically transmitted MIPs, were found to be infected at delivery, whilst their infants were HIV-1 negative at birth. The infants of these MIPs were determined to be infected at either 2 months (pairs 2617, 2669, 1449 and 2873) or 4 months (1084) after birth. Maternal samples collected at delivery and infant samples collected at the first post-partum HIV-1 PCR-positive time point were defined as baseline specimens, whilst for the other two MIPs (pairs 2660 and 834), which were followed longitudinally from delivery, mothers and infants were found to have seroconverted at the same follow-up time point after birth. MIPs 2660 and 834 were found to be infected at 18 and 4 months after birth, respectively, when the baseline specimens were obtained. These two MIPs were defined as acutely infected MIPs. The baseline HIV-1 serological status of the mother was determined by two rapid assays, Capillus (Cambridge Biotech) and Determine (Abbott Laboratories). Positive serological results were confirmed by immunofluorescence assay, as described previously ([Mantina et al., 2001](#)).

PCR, cloning and expression of Env derived from patients.

To obtain the pro-viral HIV-1 env gene, genomic DNA was extracted from uncultured PBMCs for all subjects except mother 1084. For mother 1084, the env gene was amplified from placental tissue as PBMCs were not available. The env sequences of our samples were generated previously

from pooling of several diluted PCRs rather than from one bulk PCR. We eliminated potential recombinants, and our previous studies showed that the number of unique sequences was large and was similar at each time point analysed ([Hoffmann et al., 2008](#); [Zhang et al., 2006](#)). This suggests that pooling of several individual PCR products was adequate to prevent virus template resampling in our study. Nested PCR was used to amplify a 1100 bp fragment spanning the V1–V5 region of env. The primers and PCR parameters used were as described previously ([Zhang et al., 2006](#)). Amplified fragments were cloned into a pGEM-T Easy vector (Promega) and sequenced in both directions with dideoxy terminators (ABI BigDye kit). A total of 20–40 clones were sequenced for each sample to obtain a representative measurement for the diversity of the viral population genotypes.

To obtain the Env expression constructs, the Env V1–V5 region was amplified from the pGEM-T Easy vector using primers containing restriction enzyme sites DraIII and AvrII: sense primer C-DraIII (5'-TGACCCCACTCTGTGTCACCTTA-3') and antisense primer C-AvrII (5'-CTATTCCTAGGGGCTTAATTTCTACCACTT-3'). The PCR products were subcloned into a shuttle vector, pSP72 NLA/S/Av (env gene of strain NL4.3 modified with AgeI/SbfI/AvrII for cloning purposes), using the restriction enzyme sites DraIII and AvrII. Finally, the patient V1–V5 regions of env were cloned into the Env expression vector pSRH NLA/S/Av, which was generated by modifying the mammalian expression vector pSRH containing an SV40 promoter and reading frames for NL4-3 Tat, Rev and Env (kindly provided by Dr Eric Hunter, Emory University, GA, USA). All the patient-derived chimaeric Env expression constructs were first screened for biological function using a fusion assay ([Derdeyn et al., 2004](#)). Between four and seven clones from each of the pools of sequenced Env clones from

each sample were selected for the fusion analysis. The Env clones were selected from each MIP based on the following criteria: (i) whether the Env clones were functional; (ii) the length of the Env V1–V5 fragment; (iii) the branch length of each Env in the phylogenetic tree; and (iv) the number of putative N-linked glycosylation sites. To eliminate the possibility that the clones selected for the analysis could be outliers, we then calculated the divergence for each selected clone of the MIP as the genetic distance between any sequence and the most recent common ancestor of the total previously analysed archived virus sequences. This showed that divergence from each selected Env was within the range of the characterized population and that no outlier of divergence was used in our analysis ([Kong et al., 2008](#)).

#### Cells and cell cultures.

TZM-bl cells and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % FBS, penicillin ( $50 \text{ U ml}^{-1}$ ) and streptomycin ( $50 \mu\text{g ml}^{-1}$ ).

#### Fluorescence microscopy assay for cell–cell fusion.

The assay was based on calculation of dye-transfer events between fluorescently labelled target and effector cells, as described previously ([Cohen & Melikyan, 1998](#); [Muñoz-Barroso et al., 1998](#)). COS-1 cells expressing Env (designated the 'effector cells') were labelled with the cytoplasmic marker CMAC (Molecular Probes). TZM-bl cells (the 'target cells') were co-labelled with the cytoplasmic marker CMFDA (Molecular Probes) and DiI (Molecular Probes). Briefly, a confluent monolayer of COS-1 cells on a poly-L-lysine (Sigma)-coated slide ( $1 \times 10^5$  cells) was washed twice with PBS and incubated for 30 min at  $37^\circ\text{C}$  with DMEM containing

30  $\mu$ M CMAC. The TZM-bl cells ( $3 \times 10^5$  cells) were co-labelled with 5  $\mu$ M CMFDA and 1  $\mu$ M DiI in DMEM. To induce fusion, fluorescently labelled effector cells and target cells were then co-cultured at 37 °C for 3 h. The fusion events were assessed by fluorescence microscopy. For each sample, fusion events from at least five random different fields were collected, and the data were analysed by overlaying the images using Image-Pro Plus software. The extent of fusion was calculated as the ratio of fused cells (positive for all three dyes) to the sum of fused cells and the number of effector–target cell pairs that did not fuse ([Abrahamyan et al., 2003](#)).

#### Luciferase reporter gene fusion assay.

COS-1 cells ( $1 \times 10^5$ ) were transfected with Env plasmid in 12-well plates using polyethyleneimine (Sigma). Twenty-four hours later, the transfected cells were co-cultured with TZM-bl cells ( $3 \times 10^5$ ) for another 24 h. The cells were then rinsed with PBS, lysed with lysis buffer (Promega) and centrifuged at 13 201 g for 3 min. The clarified lysate (5  $\mu$ l) was mixed with luciferin reagent (Promega) and the luciferase activity was measured using a luminometer according to the manufacturer's instructions (Promega).

#### Cell-surface expression of Env glycoprotein.

COS-1 cells were transfected with the Env expression vectors. At 48 h post-transfection, the cells were harvested using PBS supplemented with 0.5 mM EDTA, 0.5 mM EGTA and 180 mM glucose. The cells were fixed for 30 min at 4 °C in 4% paraformaldehyde (in PBS, pH 7.2). The fixed cells were then incubated with primary antibody (anti-HIV Ig; National Institutes of Health) and washed extensively, and binding of the primary antibody to the cells was detected with Alexa Fluor 488-conjugated goat anti-human antibody (Invitrogen). The mean fluorescence intensity (MFI) and percentage of

fluorophore-positive cells were detected using a FACSCalibur system (BD Biosciences).

#### Western blot analysis of HIV-1 proteins.

For analysis of the expression of Env proteins, the transfected COS-1 cells were lysed with NP-40 lysis buffer [0.5 % NP-40, 0.1 % Triton X-100, 0.1 % sodium deoxycholate, 10 mM Tris/HCl (pH 8), 150 mM NaCl, 1 mM EDTA] containing 1 % PMSF for 30 min on ice ([Furuta et al., 2006](#)). After centrifugation at 10000 g for 10 min at 4 °C, the supernatant was analysed by SDS-PAGE (10 % acrylamide) and immunoblotted using sheep anti-HIV-1 gp120 polyclonal antibody (National Institutes of Health). Primary antibodies were detected with HRP-conjugated rabbit anti-sheep secondary antibody (ZSGB-BIO), and the gp160 and gp120 bands were visualized by chemiluminescence ([Lassen et al., 2009](#)).

#### Acknowledgements

We greatly appreciate the gift of pSRHS expression vector from Dr Eric Hunter (Emory University). TZM-bl and anti-HIV-1 gp120 polyclonal antibody were obtained from the NIH AIDS Research and Reference Reagent Program. This study was supported in part by the National Natural Science Foundation of China (30970162) and Tianjin Municipal Science and Technology Foundation (08ZCGHHZ01800) to X.K. and US Public Health Service grant CA75903 and Fogarty Training grant sD43 TW001492, P30 GM103509 and T32 AIO60547 to C.W. X.K. was a Fogarty Fellow, and M. W. was supported by the NIH under a Ruth L. Kirschstein National Research Service Award from the National Institute of Allergy and Infectious Diseases.

## Footnotes

A supplementary table is available with the online version of this paper.

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**Table 1. . Summary of clinical information for the MIPs included in this study**

NA, Not applicable.

Subject	Age (years)*	Birth weight (g)	Mode of delivery	Breast- feeding	Time point analysed (month)†	No. of clones analysed
MIP 1084						
Mother	28	NA	NA	NA	M0	3
Infant, male	NA	3400	Vaginal	Yes	6	3
MIP						

2617

Mother	20	NA	NA	NA	M0	3
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Infant, female	NA	2560	Caesarean section	Yes	2	3
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MIP  
2669

Mother	19	NA	NA	NA	M0	3
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Infant, male	NA	3260	Vaginal	Yes	2	3
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MIP  
2873

Mother	31	NA	NA	NA	M0	4
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Infant, female	NA	3080	Vaginal	Yes	2	3
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MIP  
1449

Mother	30	NA	NA	NA	M0	2
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Infant,	NA	2540	Vaginal	Yes	2	2
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male

MIP

2660

Mother	21	NA	NA	NA	18	3
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Infant, male	NA	2760	Vaginal	Yes	18	2
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MIP  
834

Mother	26	NA	NA	NA	4	3
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Infant, male	NA	3400	Vaginal	Yes	4	2
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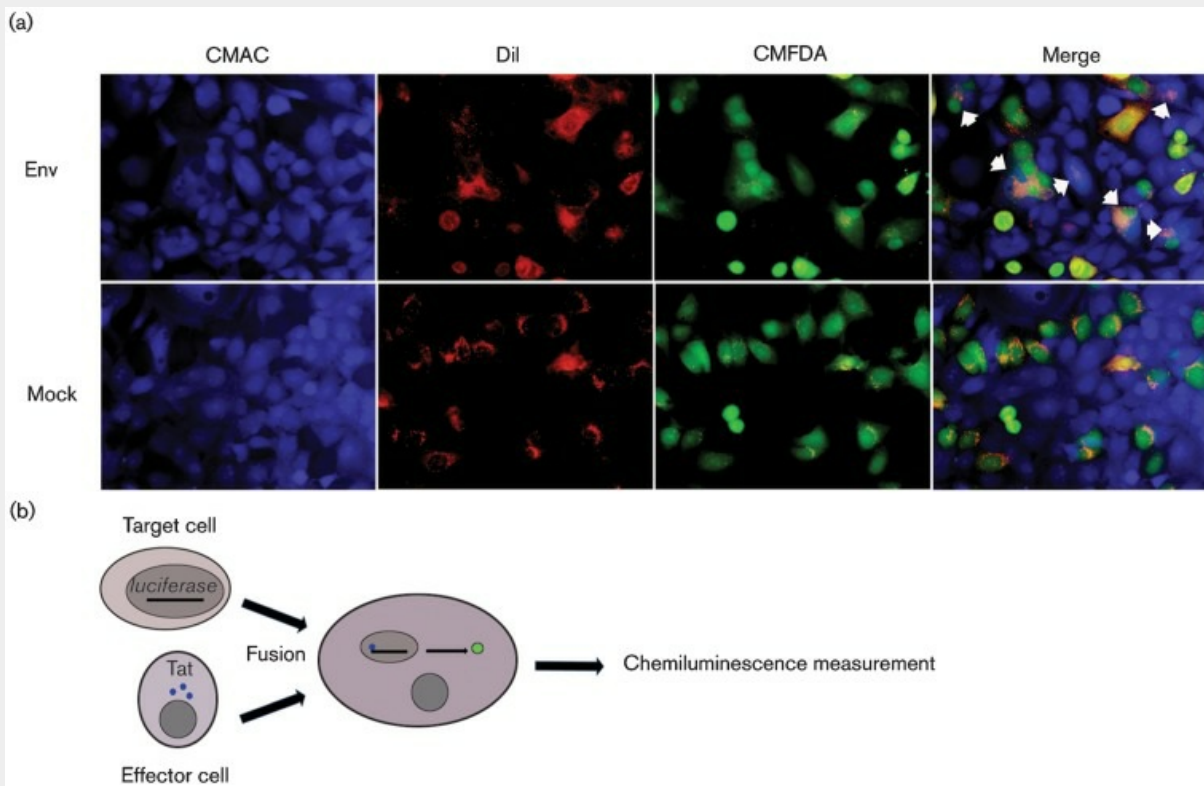
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\*. Age of the mother in years at the time of delivery.

†. M0, Maternal samples at delivery were defined as baseline. The baseline samples of other subjects were referred to the first HIV-1 PCR-positive time point, as indicated in months after infant birth.

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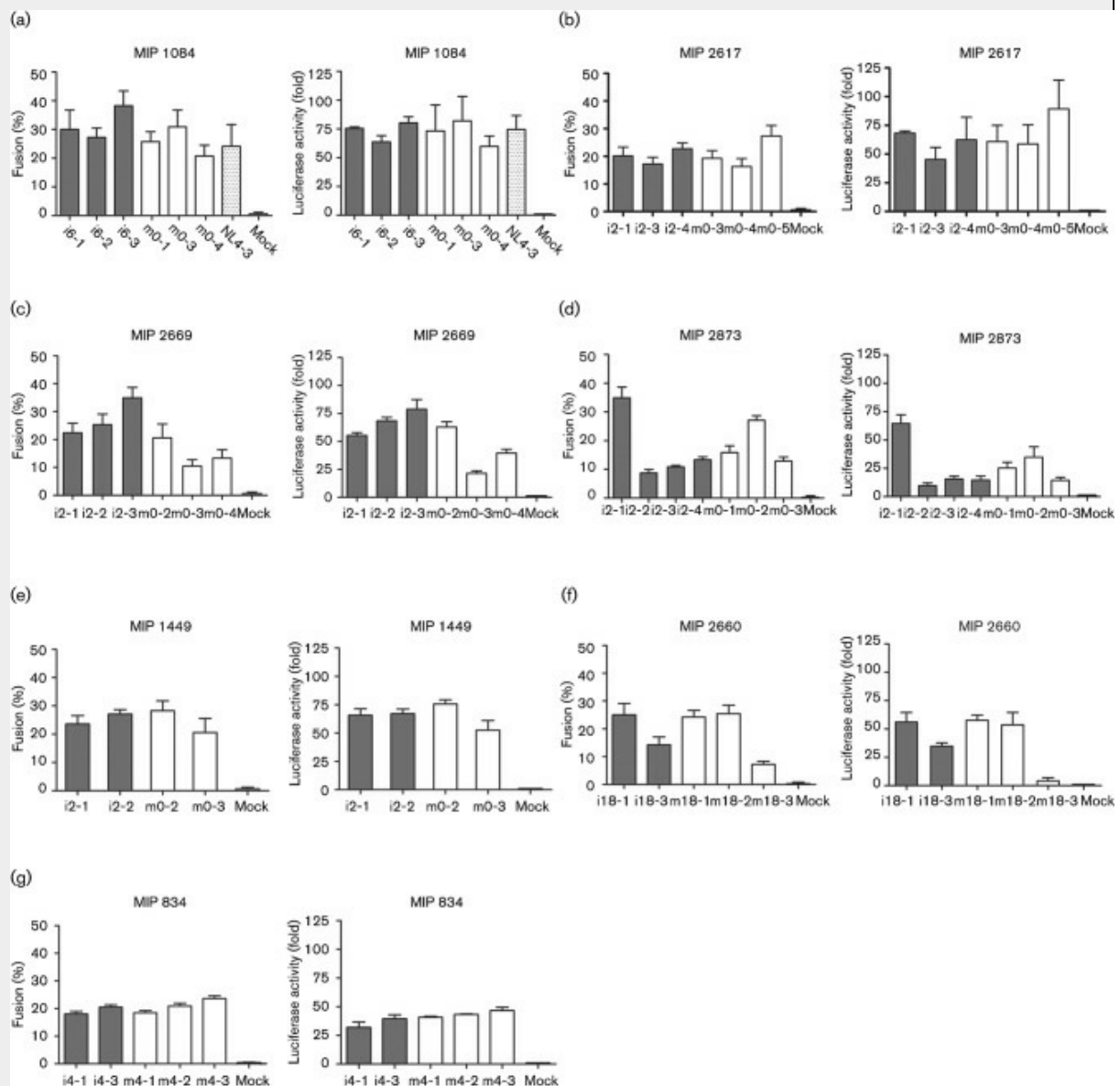


**Fig. 1. .**

Fusion assays. (a) Fluorescent microscopic images of cell–cell fusion mediated by Env using a dye-transfer-based assay. Effector (COS-1) cells expressing Env were labelled with CMAC (blue). Target (TZM-bl) cells were co-labelled with Dil (red) and CMFDA (green). Fluorescence images for each dye were acquired as shown. Fused cells are depicted by arrowheads. (b) Schematic representation of the reporter gene-based cell–cell fusion assay. TZM-bl cells were used as target cells and luciferase activity was measured to indicate fusion.

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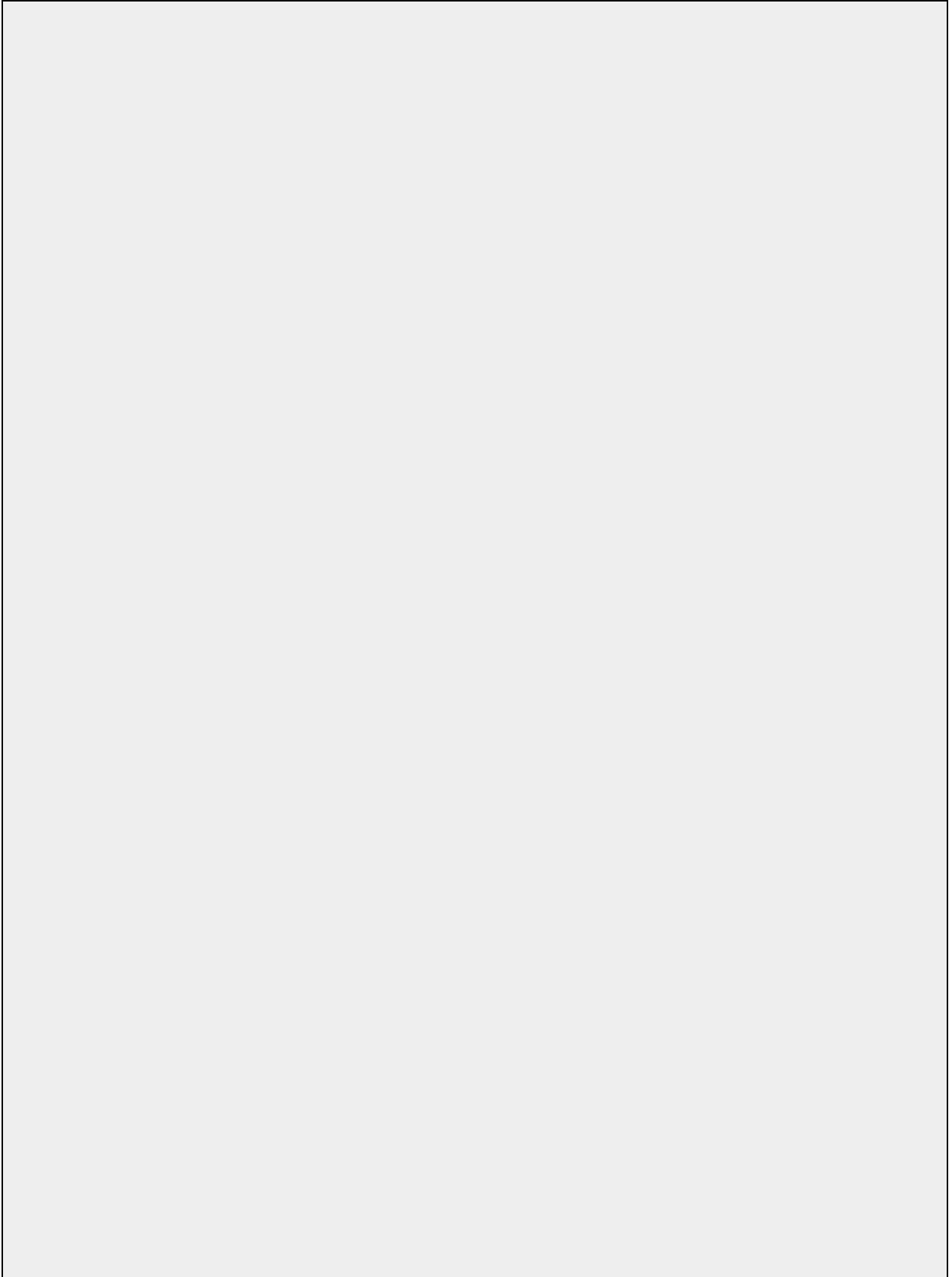
**Fig. 2. .**

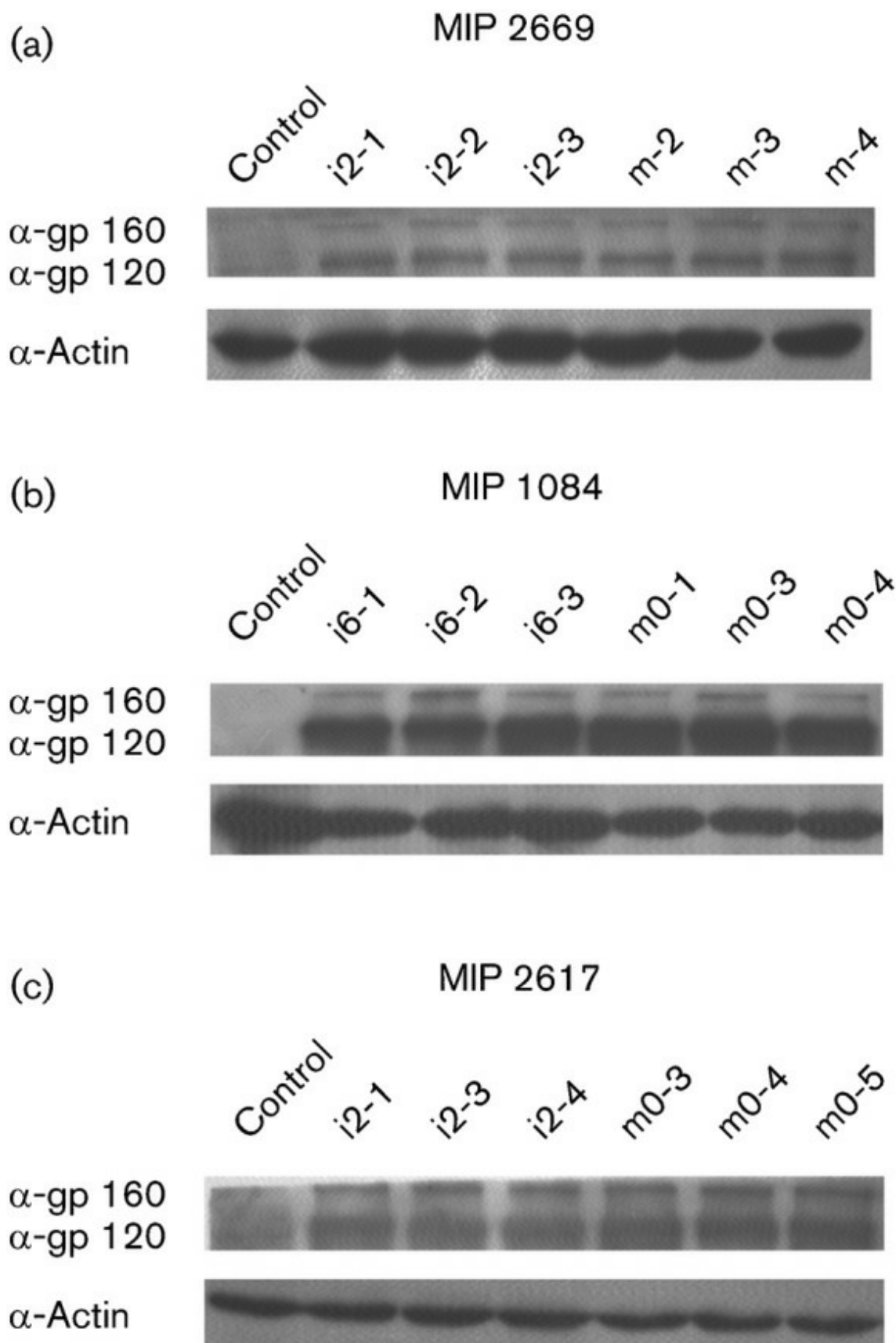
Comparison of the fusion efficiency between the infant and maternal variants from MIP 1084 (a), MIP 2617 (b), MIP 2669 (c), MIP 2873 (d), MIP 1449 (e), MIP 2660 (f) and MIP 834 (g) using a dye-transfer-based assay (left graphs) and a reporter gene-based assay (right graphs). COS-1 cells were transfected with Env-expressing plasmids and used as effector cells. TZM-bl cells were used as target cells. Maternal samples were collected at the delivery, indicated as m0. Infants samples were collected at the time of the first HIV PCR-positive result after birth, which for most infants was at 2 months after birth (i2), for some was at 4 months (i4) and for some was at 6 months (i6). The number after the dash indicates the clone number. Results

are shown as means $\pm$ SD from three independent experiments.

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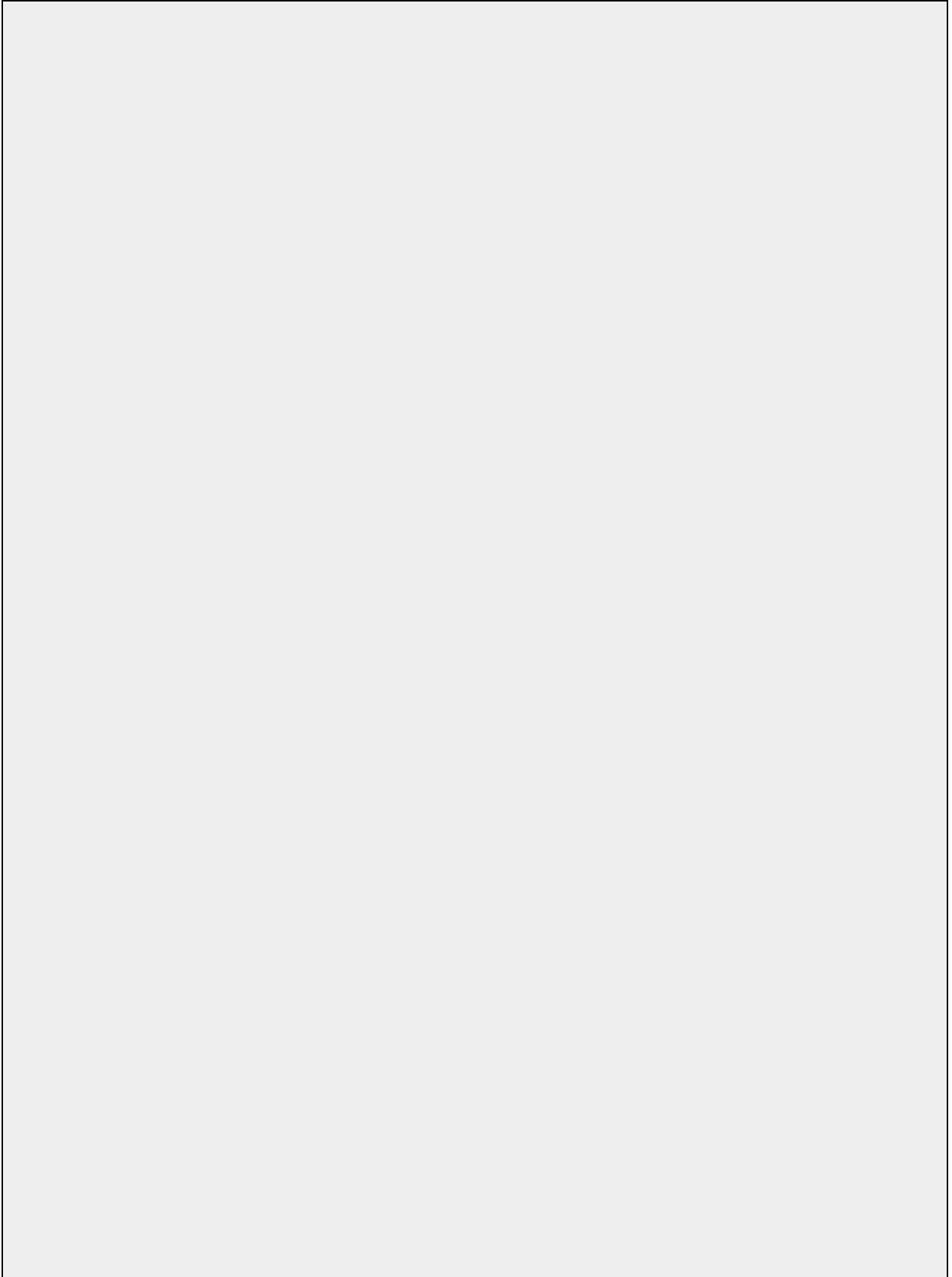


### **Fig. 3. .**

Expression profiles of Env glycoproteins containing the V1–V5 regions derived from MIP 2669 (a), MIP 1084 (b) and MIP 2617 (c). COS-1 cells transiently transfected with simian virus 40 (SV40)-based Env expression vectors containing the V1–V5 regions derived from patients were collected at 48 h post-transfection, lysed and analysed by SDS-PAGE. Detection of actin was used as a loading control.

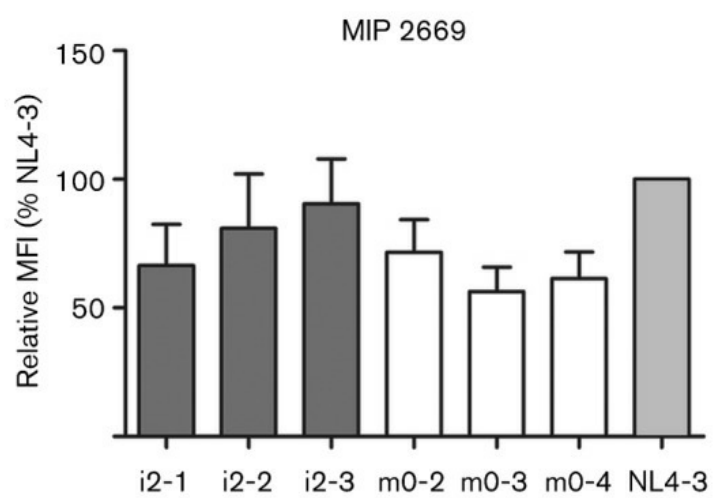
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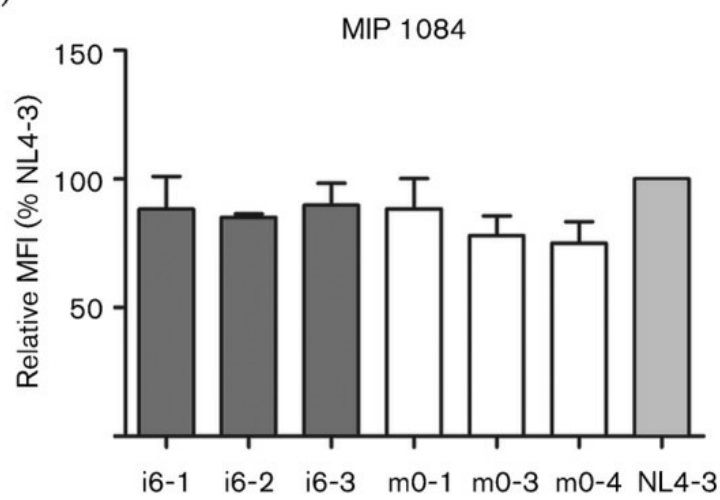




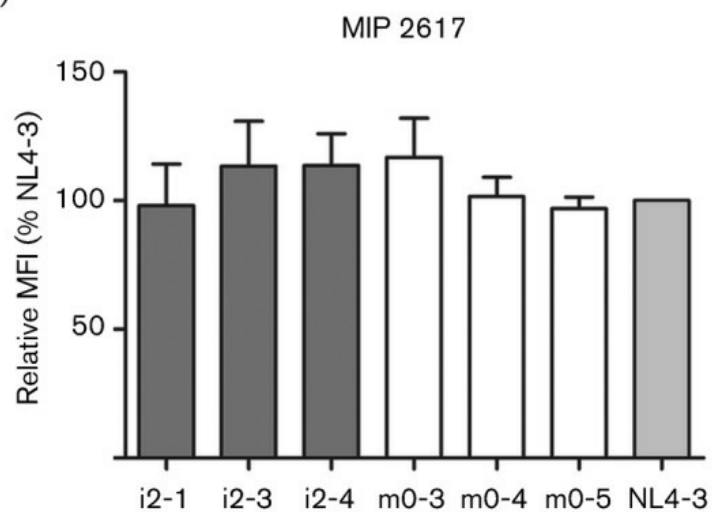
(a)



(b)



(c)

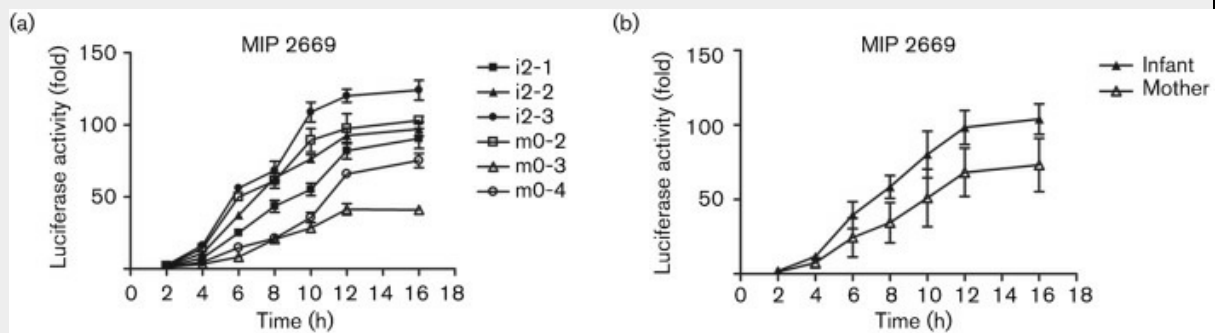


#### **Fig. 4. .**

Cell-surface levels of Env glycoprotein containing the V1–V5 regions derived from three chronically infected MIPs. COS-1 cells transfected with Env expression plasmids containing the V1–V5 regions derived from patients were fixed at 48 h post-transfection, labelled with anti-gp120 HIV antibody and analysed by flow cytometry. The surface levels of Env derived from MIP 2669 (a), MIP 1084 (b) and MIP 2617 (c) are shown as the relative mean fluorescence index (MFI) compared with that of strain NL4-3 Env. Results are shown as means $\pm$ SD from three independent experiments.

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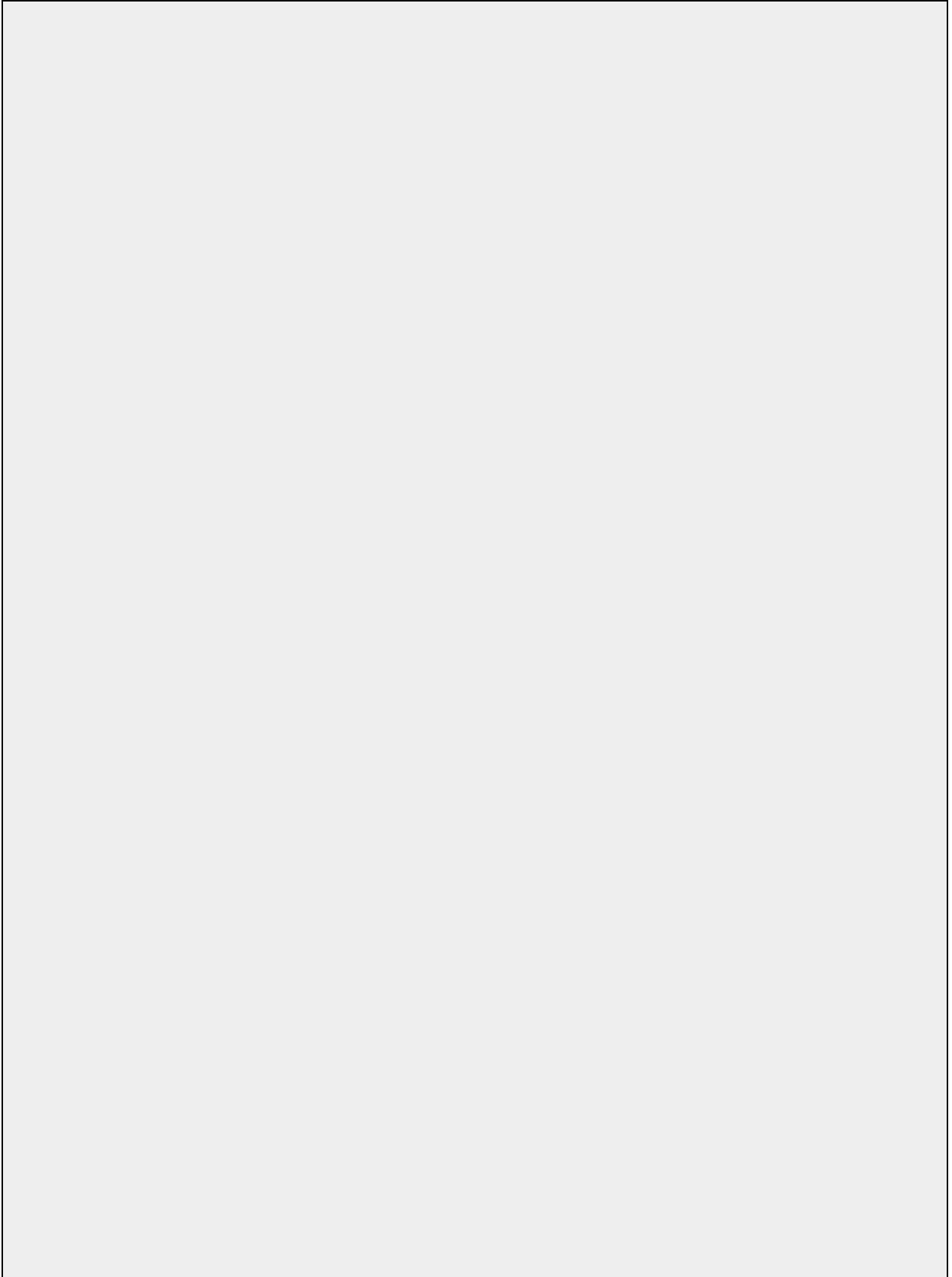


**Fig. 5. .**

Kinetics of cell-cell fusion mediated by the V1-V5 regions of infant and mother derived from MIP 2669. COS-1 cells transfected with Env containing the V1-V5 regions from chronically infected MIP 2669 were mixed with TZM-bl indicator cells and co-cultured for 2, 4, 6, 8, 10, 12 and 16 h before measuring the luciferase activity. (a) The relative luciferase activities of each infant and maternal variant were calculated at each time point and plotted as a single line. Results are shown as means $\pm$ SD of at least three independent experiments. (b) Relative luciferase activities of all maternal and infant variants. Results are shown as means $\pm$ SEM of infant and mother.

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m0-2 CSNASNASNVNATNHNATNH-----NTIKEEMKNC SFNATTEL RNKKKK  
m0-3 .....---.....D....  
m0-4 .....-...S.ASDVNAT.....D....  
i2-1 ...VNATH.DTIKEGMN-----D.N..  
i2-2 ...VNATH.DTIKEEMN-----D....  
i2-3 ...VNATH.DTIKEEMN-----D....

#### V1/V2

m0-2 EYALFYRLDIVPLNASGVNSSEYRLINCNTSTITQACPKVSFDPIPIHYC  
m0-3 .....E.D.....  
m0-4 .....  
i2-1 .....N.....  
i2-2 .....  
i2-3 .....

m0-2 APAGYAILKCNNKTFNGTGPCNNVSTVHCTHGIKPVVSTQLLLNGSLAEK  
m0-3 .....  
m0-4 .....E  
i2-1 .....  
i2-2 .....  
i2-3 .....

m0-2 DIVIRSENITNNVKV IIVHLNKSVEIMCTRP GNNTRRS MWIGPGQAFYAT  
m0-3 .....K.....  
m0-4 N.....V.....K.IR.....  
i2-1 .....K.....  
i2-2 .....K.....  
i2-3 .....K.....

#### V3

m0-2 GDIIGDIRQAYCNISKDQWNETLNNVRKELNKYFPDKTIKFEP AIEGGDL  
m0-3 .....  
m0-4 .....N.....H.....NK.....K.EK.....N.....K.....  
i2-1 .....  
i2-2 .....  
i2-3 .....R.....

#### V4

m0-2 EITHSFNCRGEFFYCNTSKLF-NDTYMKTNSTEGASNITTITCRIKQII  
m0-3 .....N.....R.....K.....P.K.....  
m0-4 .....R.-.....-E.....P.K.....  
i2-1 .....-.....A.....  
i2-2 .....-.....  
i2-3 .....-.....

#### V5

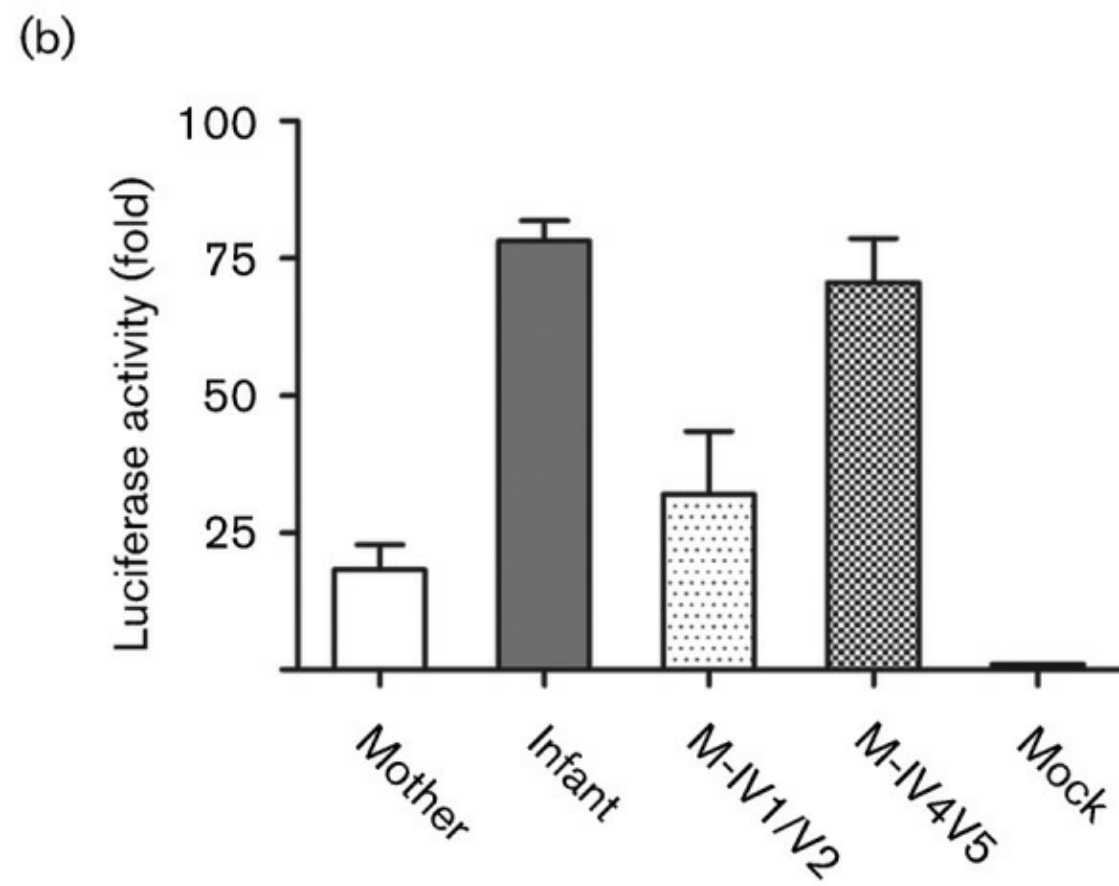
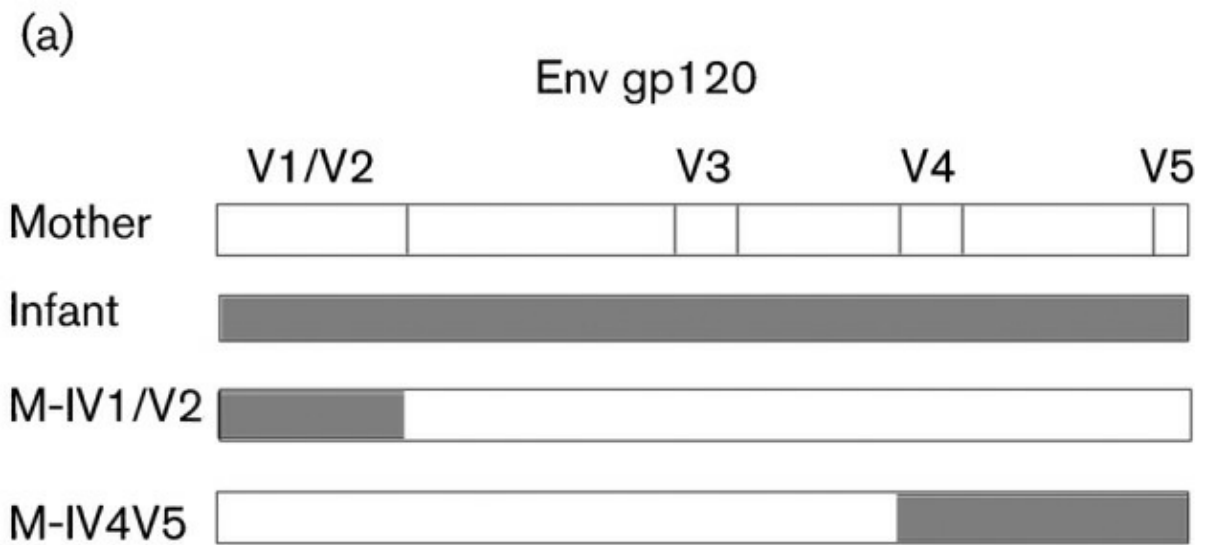
m0-2 NMWQGVGRAMYAPPIAGNITCTS NITGLILTRDGGDNN--NSTETFRPA  
m0-3 .....A.....N..ET.T.....  
m0-4 ..S...Q.....--R.....  
i2-1 .....N..ET.T.....  
i2-2 .....N..ET.T.....  
i2-3 .....N.---T.....

## **Fig. 6. .**

Alignment of deduced amino acid sequences of the Env glycoprotein spanning the V1–V5 regions derived from chronically infected MIP 2669. Amino acid identity (.), insertions/deletions (–) and substitutions are indicated.

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## **Fig. 7. .**

Identification of V4–V5 regions responsible for the high fusion efficiency of infant variants compared with the maternal variants in MIP 2669. (a) Schematic representation of chimaeric Env constructs of MIP 2669. (b) Comparison of the fusion efficiency of wild-type mother and infant and chimaeric Env using a reporter gene-based assay.

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